HUMAN AHH STUDIES

Development of Rapid Assay Procedure

and

Collaborative Studies with University of Southern California

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Vincent L. Ruwet
Vice-President, Contracts
and Administration

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TO: Council for Tobacco Research
110 East 59th Street
New York, New York 10022

FROM: Microbiological Associates, A Division
of Dynasciences Corporation
4733 Bethesda Avenue
Bethesda, Maryland 20014

DATE: September 3, 1974

Prepared by

Richard E. Kouri, Ph.D.
Project Director

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I. Introduction

The Council for Tobacco Research, is engaged in a program to reduce carcinogenic and other hazards of smoking to human health. To this end, the determination of relative risk of particular populations to smoke-associated neoplasias is one such goal. This proposal outlines specific approaches that should provide a major step in achieving this goal. At the end of the proposed 14 months of experimentation, one or more standard assay procedure for the determination of levels of aryl hydrocarbon hydroxylase (AHH) in human populations will be available. Using such assay procedures, the relationship between levels of AHH inducibility and at least eight different cancers (including cigarette-smoke-associated lung carcinomas) will be established.

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II. Background.

Aryl hydrocarbon hydroxylase (AHH) is the name given to one of the multi-component microsomal-bound enzymes (1,2). The actual steps involved in this oxidative metabolism are unknown; however, the best guess is that the substrate combines with the oxidized form of a carbon monoxide sensitive hemoprotein called cytochrome P-450. The substrate-cytochrome P-450 complex is then reduced by an electron donated by NADPH-cytochrome c reductase, to form a reduced substrate-cytochrome P-450 complex. This complex in turn reacts with molecular oxygen to form a reduced substrate-cytochrome P-450-0_2 complex. A second electron is then added to this complex to yield an active oxygen intermediate which decomposes with the formation of the product and the oxidized P-450. The product of this reaction, if polycyclic aromatic hydrocarbons (PAH) are substrates, are probably epoxides (3,4). These epoxide intermediates then:

1. Rearrange spontaneously to form phenols,
2. Are enzymatically metabolized to the dihydrodiol via the enzyme epoxide hydrase, or
3. Are enzymatically conjugated with glutathione using the enzyme glutathione conjugase.

These enzymes have two properties which make them uniquely important to the study of chemical carcinogenesis. First, metabolism of many substrates (especially PAH) does not necessarily result in detoxification, but rather are converted to water-soluble forms via transient chemically-reactive intermediates that are both cytotoxic (5,7) and carcinogenic (8,9). Second, this enzyme system is inducible by certain substrates, and this induction results in the enhanced metabolism of many foreign compounds (10). This latter property is important because, if these metabolic pathways for PAH are etiologically important in the initiation of chemical carcinogenesis, any marked changes in rates of formation of the water-soluble metabolites (e.g., epoxides, phenols, dihydrodiols and glutathione conjugates) or covalently bound metabolites, should affect the host's susceptibility to cancer.

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In the house mouse, Mus musculus, not only is AHH in-

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duced by treatment with certain substrates, but this inducibility also is host gene regulated. Treatment with phenobarbitol (PB) increases the metabolism of most of the drug substrates in every strain of mouse tested (11). Treatment with 3-methylcholanthrene (MCA) increases the metabolism of very few substrates and in only particular strains (12). These differences probably result from the fact that PB causes a rapid nonspecific proliferation of constitutive AHH (13), while MCA induces a new spectrally distinct cytochrome called P^1-450 or P448 (14,15) which has different substrate specificities. The ability to respond to MCA (but not PB) segregates as a single autosomal gene in crosses involving the C57BL/6 (B6) and DBA/2 (D2) strains of mice (12, 16, 17, 18). We proposed this locus be designated Ah; the allele carried by the B6 mouse (inducible) is Ah^b, and the allele carried by the D2 mouse (noninducible) is Ah^d. Following treatment with MCA, the differences between the AHH levels in various tissues of Ah^b/Ah^b or Ah^d/Ah^d mice are 2-80 fold greater than that of Ah^d/Ah^d animals (16,19). Thus, one can evaluate tumor susceptibility among litter-mates in which the presence or absence of AHH induction is expressed in their tissues. With the use of such a model, other nonspecific strain differences - such as characteristic mouse strain differences involving immunology, latent viral infections, nutrition, hormones, stress, or levels of other enzymes - will be theoretically cancelled.

Using this model system, we have reported that segregants carrying the Ah^b allele are approximately 12 times more sensitive to MCA induced fibrosarcomas than animals homozygous for the Ah^d allele (20,21). Thus, it seems likely that the types of metabolites, or just the quantity of these metabolites determined by this novel "inducible" enzyme play a major role in determining the susceptibility of mice to chemical carcinogenesis.

The human situation may be analogous to that described in mice. AHH induction is variable in humans (22); however, three distinct groups are observed: low, intermediate and high inducibility (22,23). Results suggest a single locus of genetic control, with gene frequency of the low and high alleles

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being .717 and .283 respectively. To date, the information about phenotypic variations of AHH is limited to Caucasians. There is no information available, of which we are aware, indicating the frequency distribution of AHH among Blacks, Orientals American Indians, or specific ethnic populations. In this limited system, the only disease shown to harbor a possible association with AHH has been the published work by Kellerman, et al, demonstrating an association between AHH, cigarette smoking and bronchogenic carcinoma (24). This same group has data showing a similar association with carcinoma of the colon though this is unpublished, but was told to our group and presented in a very preliminary manner at the recent meetings of the American Society of Human Genetics at Atlanta, Georgia. This result suggests that PAH are important causes of cancer in humans and that, as in mice, those individuals with a heightened ability to metabolize PAH are more susceptible to the chemically-induced or "spontaneous" cancers.

These points are of extreme interest to cancer epidemiologists since it is well-known that cancer is not uniformly distributed in the population. Rather, there are marked variations in all specific anatomic varieties of cancer. Many of these variations correlate with different geographic areas, racial and ethnic groups and, in addition, familial factors, habit patterns, and occupational factors condition these variations significantly. In light of the limited knowledge available on AHH in differing populations in different clinical settings, as well as the limited knowledge with respect to disease associations, it would seem compelling that studies be developed to extend knowledge in these specific areas.

The major assays used to detect AHH activity both in vitro and in vivo have been modifications of the original one described by Nebert and Gelboin (25). The assay is based on the ability to spectrophotofluorometrically detect one of the phenolic metabolites of benzo(a)pyrene (BP), 3-hydroxybenzo(a)-pyrene (3-OH BP). The material being assayed is added to a small volume of buffer containing NADPH and Mg^{++}. The assay is initiated by the addition of BP and allowed to run at 37°C for 10 to 30 minutes. Cold acetone-hexane is added to stop

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the reaction and to extract most of the metabolites as well as the unmetabolized BP. A sample of the hexane-acetone phase is extracted with 1N NaOH and the amount of 3-OH BP in the alkaline phase is determined. The assay is only limited to the resolving power of the fluorometric instrument; the limit being about 0.5 pmoles 3-OH BP per ml, or about 0.01 pmoles 3-OH BP per mg protein per minute. Advantages of this assay are that it is capable of monitoring both constitutive and induced levels of AHH, is highly sensitive and is relatively inexpensive. Disadvantages are the low levels of AHH which need to be determined in human studies are close to the sensitivity limits of this assay and extreme care must be taken to avoid wide day-to-day fluctuations. Factors such as pH of the buffer, temperature of the assay, purity of the solvents, or cleanliness of the glassware must be controlled. A modification (26) of this assay has been used to detect the levels in human lymphocytes. When care is taken, this assay can reproducibly detect those low levels of AHH, however, some of these modifications also possess inherent problems. For example, the lymphocytes must be activated by a mitogen and this activation step can be influenced by the physiological status of the donor and could be changed if the donor is on medications such as steroids, aspirin or immunosuppressants. This activation step is also very sensitive to changes in pH, temperature or types of growth medium (27). Another disadvantage is that the assay now takes 5 days to complete. This definitely limits the number of assays that can be performed. This assay can measure the AHH levels in human alveolar macrophages (28); however, the levels observed are even lower than those observed in cultured lymphocytes.

Three main problems must be worked out before this assay can be used in a large scale screening study of human population. First, a reproducible source of cells must be made available. Second, procedural modifications must be done so as to lower the rather high non-specific fluorescence observed in the zero-time control cultures. Third, the number of tests capable of being performed must be greatly increased.

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Since previous studies have shown that the small lymphocyte is capable of giving reproducible AHH inducibility results (22, 26), this cell is probably the one of choice. The major ways to isolate lymphocytes are ammonium chloride-DEAE dextran precipitation and gradients, such as ficoll-hypaque. We feel the latter technique has certain advantages - mainly it yields a clean, relatively pure, "band" of small lymphocytes. In our laboratory, this "band" consists of cells in which greater than 85% are small lymphocytes. One way to express enzyme activity is units of activity per a certain number of cells (e.g. 10^6 cells). Thus, the more accurately one can determine the number of cells, the more reproducible the results. The isolated lymphocytes must be activated in vitro by mitogens, such as pokeweek mitogen (PW) or phytohemagglutanin (PHA). Doses of PW and PHA approximating 1% seem to give optimal activation (29). This activation step is very sensitive to changes in pH, temperature, or types of growth medium (27). To this end, large lots of medium (RPMI - #1640), fetal calf serum, mitogens, and antibiotics must be purchased, pretested, and stored. Only then can a standard source of cells be relatively assured. The use of the small lymphocyte may have a secondary advantage because it is metabolically quiescent in vivo and is activated in vitro. Many in vivo physiological changes, such as drug treatment, diseases, exposures to pollutants, etc., may only negligibly affect the lymphocyte.

For the standardization of the assay, the optimal pH, temperature, incubation time, cell concentration, and buffer will be determined. The effect of solvent and/or various inducers will be tested. The chemical, 7,8-benzoflavone will probably be the inducer of choice because it can maximally induce AHH and is not carcinogenic.

The assay itself has inherent problems because of two major facts:

a. The level of enzymes observed in the cultured lymphocytes, although discernible, approaches the sensitivity limits of this assay, and

b. Levels of AHH in the three human subpopulations are

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not that different.

The latter problem is exemplified by the fact that inducibility levels (relative increases over vehicle-treated control levels) of 2, 3, and 4 represent the ideal low, intermediate and high AHH inducibility subgroups of the human population. These two problems can be largely rectified if one concentrates on one particular facet of the assay - the relative difference between the non-induced (constitutive) AHH activity and zero time control values. The constitutive activity is that level of enzyme observed in mitogen-activated, vehicle-treated, control cultures. The zero time control contains the complete reaction mixture plus cells and BP, but in which cold acetone-hexane was added before incubation. If the constitutive activity is not sufficiently greater than this zero time control, then small deviations in the zero time activity will greatly affect the AHH inducibility ratios. For example, typical results may be:

AHH (Fluorescent units)	
Constitutive	Induced
0.8	1.6

If zero time is 0.3 then inducibility is $\frac{1.6-0.3}{0.8-0.3}$ or 2.6

If zero time is 0.1 then inducibility is $\frac{1.6-0.1}{0.8-0.1}$ or 2.1

Therefore, this individual could be either a low inducer or an intermediate inducer, depending on which zero time was observed. The major ways to increase the difference between the zero time and constitutive fluorescence values are:

a. The use of more cells;

b. Use of longer incubation periods (the assay is linear for up to 1 hour);

c. Use of carefully cleaned glassware (soap with no brighteners), and

d. Use of highly purified reagents.

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For the standard assay, negligible zero time fluorescence relative to constitutive activity (<10%) will be the goal.

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III. Experimental

A. Use of fresh blood.

Recent information from our laboratories has demonstrated that blood can be kept at room temperature (or shipped at room temperature) for at least 24 hours and the lymphocytes can still be activated and assayed. The following protocol has been tentatively established:

1. Collect 20 ml venous blood (in heparinized tubes) between 11:00 AM and 1:00 PM (PCT);
2. Send heparinized tubes of whole blood via regularly scheduled airlines from L.A. International Airport to Dulles International Airport. American Airlines Flight #110 at 2:30 PM is probably the best. Samples arrive at 11:30 PM (EST) and are kept at room temperature until the next morning for pick up at the Airport at 8:00 AM and brought to the laboratory;
3. At that time, dilute blood 20% with medium;
4. Layer 9 ml aliquots of diluted blood onto 6 ml ficoll-hypaque solution;
5. Centrifuge 45 minutes, 1200 xg;
6. Collect lymphocyte "band" and wash twice with medium;
7. Count cells in an automatic counter, adjust to 0.5 X 10^6/ml, and add 1.0 ml per incubation tube;
8. Medium will be RPMI #1640 supplemented with 20% fetal calf serum, antibiotics, 1% PHA and 1% PM;
9. Incubate 72 hours at 37°C, 5% CO_2;
10. Add 0.01 ml of 0.75mM 3-methylcholanthrene or 7,8-benzoflavone to certain cultures and 0.01 ml acetone to controls;
11. Twenty-four hours later, count cells, pellet at 1,000 xg for 10 minutes and resuspend cells in a 0.1M tris-chloride buffer (pH 8.5) containing 2.7 mg bovine serum albumin, 0.8 µg NADPH, and 0.006M $MgCl_2$ at a concentration of 4 x 10^6 cells per ml.
12. Add 80nMoles BP in a total of 0.010ml acetone to each tube and incubate for 45 min at 37°C in air with shaking.

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13. Stop the assay with cold acetone-hexane (1:3);
14. After shaking the two phases of 37°C for an additional 10 min, extract a 3.0 ml aliquot of the upper hexane phase with 1.0 ml in NaOH;
15. Quantitate the amount of 3-OH BP in the alkaline phase in an Aminco-Bowman spectrophotofluorometer with excitation at 396nm and emission at 522nm;
16. Express data in pMoles 3-OH BP formed per min per 10^6 cells.

These procedural changes definitely limit the number of assays that can be performed. One technician can only handle about 20-30 individual patients per week.

B. Use of frozen samples.

The interposition of a freezing step in this aforementioned protocol should have the following advantages: (1) transfer and delegation of some of the early procedural steps to the "collective" laboratories, thus allowing for an increased number of assays to be performed, and (2) circumventing a possible major problem when using fresh material; that is, rapid, efficient and responsible shipping.

1. Use of frozen lymphocytes.

A freezing step can be introduced after lymphocyte isolation. The cells must be slow-frozen according to standard procedures and held at -120°C. The cells can then be shipped in dry ice containers and held until 20-30 samples have been accumulated. At this time, the cells can be thawed, cultured, activated, and assayed. The use of this freezing step should double or triple the number of assays that can be performed per week.

2. Use off frozen-activated-induced cells.

A freezing step can also be worked in after the cells have been collected, activated, and induced. The induced cells will be collected as described in steps 4-16 of the aforementioned protocol, however, the induced and control cells will be stored at -70°C in a pellet form with 0.2ml tris-HCl buffer on top of this pellet. When 100-300 frozen samples have been

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collected, all can be assayed on the same day. In this way, one central laboratory can run the assay for various "collection" centers. Large pools of lymphocytes derived from known low, intermediate and high AHH inducers could be frozen and run concomitantly with test cells. This could serve as an invaluable internal assay control. This procedural alteration could increase the number of assays performed probably by 5 to 10 fold.

C. Source of cells.

The population of Menck et al (30) appears to fulfill the criteria of accessibility as well as availability of adequate numbers of patients. The initial study will be concentrated on the lung cancer populatiom. 50-100 lung cancer patients, 50-100 hospital controls and 50-100 non-hospitalized controls will be assayed. Assays will be done at MA at a rate of 20-50 per week. A confirmatory assay under the supervision of Drs. J. Brown and R. Gordon (USC) will also be done, but only at a rather low level (about 4-10 per week). This study will also entail a limited questionnaire involvement; including history of cigarette smoking, drug exposure, occupation etc. Medical verification of the pathological lesion will also be done.

Beginning Oct-Nov, 1974, the suggested approach will concentrate on the importance of cancer association of AHH inducibility. A matched population would seem to be the best obtainable control at this level. The requirement for detailed questionnaire and multi-variant analysis of same must be incorporated to observe correlates and associations. Smoking habits, while normally considered a tertiary study variable, are included in this secondary study, since this variable has particular interest to the granting agency. The procedure for this secondary study will include one hundred patients for each of seven cancer types (total 700) and 700 to 800 controls matched for age, sex, ethnic group and smoking habits. The routine fluorometric assay discussed before will be done. However, concurrent preparations for automated analysis will be included in preparation for the tertiary level studies. The questionnaire for this study will be detailed and organized for computeriza-

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tion. This indepth level of inquiry would give operational experience and possible leads for specific indepth tertiary studies. This detailed questionnaire is compatible with the 1,500 patients projected. Areas to be emphasized in this secondary level include: 1) normal medical history; 2) detailed employment history - with master breakdown code of probable PAH, etc. exposure; 3) detailed drug history, weighted for those drugs involved in AHH; 4) detailed environmental history and breakdown code; 5) detailed smoking history; 6) detailed alcoholic consumption history; 7) other variables to be defined - possible psychological evaluations.

The questionnaire validity will be important. Analysis should be undertaken for possible correlations as the data accumulates. These correlations can be used to predict, direct, and avoid unnecessary duplication in the following studies.

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R: REDACTED MATERIAL

Soc. Sec. No [redacted]3

V. CURRICULUM VITAE - RICHARD E. KOURI

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PROFESSIONAL AFFILIATIONS: REDACTED

REDACTED

PRESENT POSITION: 1974 - present

REDACTED

REDACTED

POSITION DESCRIPTION: Development and application of in vitro and in vivo viral-chemical carcinogenesis assay systems for tobacco smoke and smoke components, and the role of viruses and chemicals in the etiology of cancer. The role of the enzyme complex, aryl hydrocarbon hydroxylase, in cancers of both animals and man.

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PRIOR
EXPERIENCE: 1972 - 1974

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1971 - 1972

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1970 - 1971

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1967 - 1970

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1966 - 1970

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Schedule C

Equipment

1.	Compu -pet 100 (automatic pipetter) (Fisher Scientific)	$1,295.00
	- parts and accessories	315.00
2.	Autocytometer II (automatic cell counter) (Fisher Scientific)	4,500.00
3.	Automatic dilutor (Fisher Scientific)	400.00
	Total	$6,510.00

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Schedule B. Other Direct Costs

Materials		
Media (MA)	$2,925.	
Blood Samples	585.	
Chemicals	5,148.	
Total		$8,658.
Expendable Supplies		
Quartz cuvettes	$1,170.	
Glassware (productions and reuseable)	2,340.	
Disposable plasticware	2,935.	
Total		$6,442.
Total Other Direct Costs		$15,100.

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R: REDACTED MATERIAL

Schedule A

Direct Labor Costs

Name & Position	Time on Project	Total hrs*	$/hr	Total $
R. E. Kouri, Ph.D. Project Director	10%	225	REDACTED	
Vacancy, Ph.D. Asst. Proj. Dir.	40%	899	REDACTED	
C. McKinney Technician	100%	2247	REDACTED	
Vacancy, Technician	100%	2247	REDACTED	
Total		5618		$26,967.
Total Direct Labor (+ 6% raise)				1,618.
TOTAL				$28,585.

*Based on 14 months (2427 hours or 2247 working hours) (Nov 1, 1974 - Dec 31, 1975).

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VI BUDGET

(Based on 14 month period from Nov 1, 1974 to Dec 31, 1975)

A.	Total Direct Labor (Schedule A)	$ 28,585.00
B.	Overhead (115% of A)	32,872.00
C.	Other Direct Costs (Schedule B)	15,100.00
D.	Travel ($500.00/professional plus three trips L.A. - Wash. D.C.)	2,500.00
E.	Total (A-D)	$79,057.00
F.	G & A (16% of E)	12,649.00
G.	Total Costs	$91,706.00
H.	Fixed Fee (10%)	10,190.00
I.	Total Cost before equipment	$101,895.00
J.	Equipment (Schedule C)	6,510.00
K.	Total Cost	$108,406.00

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